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Jon W Dudas

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PROVISIONAL APPLICATION COVER SHEET

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This is a request for filing a **PROVISIONAL APPLICATION** under 37 CFR 1.53(c).

INVENTOR(S)/APPLICANT(S)				
Last Name	First Name	Middle Initial	Residence (City and Either State or Foreign Country)	
SAMUELSSON	Göran		Umeå, Sweden	
TITLE OF INVENTION (280 characters max)				
Method for Production in Plants and Recombinant Constructs Therefor				
CORRESPONDENCE ADDRESS				
<input checked="" type="checkbox"/> Customer Number 24256 OR		Holly D. Kozlowski Dinsmore & Shohl LLP 1900 Chemed Center 255 East Fifth Street Cincinnati, Ohio 45202 USA		
ENCLOSED APPLICATION PARTS (check all that apply)				
<input checked="" type="checkbox"/> Specification	Number of Pages	6	<input type="checkbox"/> Other (specify) _____	
<input type="checkbox"/> Drawing(s)	Number of Pages	_____		
METHOD OF PAYMENT (check one)				
<input checked="" type="checkbox"/>	Applicant(s) claim(s) small entity status, 37 C.F.R. 1.27		Provisional Filing Fee Amount(s) \$80.00	
<input type="checkbox"/>	A check or money order is enclosed to cover the Provisional Filing Fee			
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Respectfully submitted,

SIGNATURE:

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☐ Additional inventors are being named on separately numbered sheets attached hereto**CERTIFICATE OF EXPRESS MAILING**

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Method for production in plants and recombinant constructs therefor

The present invention relates to production of various components, e.g. proteins, in plants according to a new method comprising targeting of any nuclear encoded component to the chloroplast/plastid stroma through the ER (endoplasmic reticulum). The invention further comprises the recombinant constructs, e.g. vectors comprising the signalling system required and optionally sequences for glycosylation or other modifications of the component, e.g. in the ER or chloroplast/plastid.

It is well established that targeting foreign components to plastids improves expression. Proteins will be used as the model component in the present application even if the invention can be utilized also with other components, e.g. peptides. Plastid targeted proteins accumulate up to 30-40% of total soluble protein of the leaf, as compared with 0.01-0.4% with cytosolic expressed proteins. It also alleviates cytosolic toxicity and other deleterious effects of the gene products. In addition, chloroplasts can process eukaryotic proteins (for instance folding, formation of disulfide bridges), which in many cases will eliminate the need for complicated and expensive *in vitro* processing of biopharmaceutical proteins in other recombinant systems (i.e., bacteria). All these reasons make the plastid targeting system the best for expressing foreign proteins in plants.

With the method according to the present invention with the ER - plastid pathway, in addition to all the advantages pointed before; optionally glycosylated proteins can be produced and accumulated in the plastid. This is of great importance for production of certain biologically active compounds to be used in therapy and diagnosis with high requirements with regard to binding and other functional characteristics. Non-glycosylated antibodies expressed in plants according to prior art methods will not be suitable for its use in patients.

The method according to the invention is suitable to be used, for instance to express/produce:

- Vaccines (such as vaccines against for example hepatitis B virus envelope protein, human cytomegalovirus glycoprotein B, Norwalk virus capsid protein, etc, etc.) because some of them are glycosylated.
- Antibodies or antibody fragments.

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- Pharmaceutical proteins such as signal peptides, protein hormones, structural proteins such as collagen, blood proteins such as serum albumin, enzymes such as secreted alkaline phosphatase, etc, etc.
- Industrial enzymes
- Consumer enzymes, such as enzymes used in washing powder.
- Enzymes that produce a secondary or new metabolite/chemical compound in the chloroplast.

A DNA construct according to the invention is introduced into a plant transiently or in a stable manner using any technique for introduction of DNA into plant cells. Such known methods include, without limitation, Agrobacterium mediated transfer, particle bombardment, electroporation, chemically induced introduction, conjugation, crossings, protoplast fusions etc. After transformation into the plant (being any plant, or green algae) individual transformants are analysed and selected. Such plants could already contain other mutations or transgenic DNA fragments that could for example change the glycosylation pattern of proteins in the plant.

Arabidopsis CAH1 (U73462) has been found to be of special interest with regard to the method of the invention as well sequences in CAH1 for construction of recombinant vectors for production of various compounds.

The protein sequence of CAH1 is:

```

MKIMMMIKLCFFMSLICIAPADAQ
TEGVVFGYKKGKNGPNQWGHINPHITCAVGKIQSPIDIQRRQIFYNHKLNSIHREYYFTNATLVNHVC
NVAMFFGEA
GDVIENKNYTLQMHWHTPSEFHLLIGVQYAAELHMVIIQAKDGSFAVVASLFKIGTEEPFLSQMKEK
LVKLKEERLKGHNHTAQVEVGRIDTRHIERKTRKYYRYIGSLTTPPCSENVSWT
II.GKVRMSKEQVELLRSLDTSFKNNSRPCQLNGRRVEMFHDHERVDKKETGNKKKKPN

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with the corresponding DNA sequence:

The open reading frame is underlined

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1 atgcagtaat ctgataaac cctccacaga gattccaac aaaacaggaa ctaaacaca
61 ag atgaagat tatgatgatg attaagctct gctctctc catgtccctc atctgcattg
121 cacctgcaga tgctcagaca gaaggagtag tctttggata taaaggcaaa aatggaccan

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181 accaatggg acacttaac cctcactca ccacatgcg ggtcggtaa ttgcaatct
 241 caattgatat tcaaaggagg caaatatit acaaccacaa attgaattca atacaccgt
 301 aatactactt cacaacgca acactagtga accacgtctg taatgttgcc atgttctcg
 361 gggagggagc agcacatctg ataataaaa acaagaacta taccttactg caaatgcatt
 421 ggcacactcc ttctgaacat cactccatg gactccaata tgcagctgag ctgcacatg
 481 tacaccaagc aaaagatgga agcittgctg tggggcaag tclctcaaa atcggcactg
 541 aagagcctt cctctctcag atgaaggaga aattggtgaa gctaaggaa gagagactca
 601 aagggaacca cacagcaca gtggaagtat gaagaatcga cacaagacac attgaacgta
 661 agactcgaaa gtaclacaga tacattgggt cactcaclac tclctctgc tccgagaacg
 721 ttcttgac calcttggc aagtgaggt caatgtcaaa ggaacaagta gaactactca
 781 gatctcatt ggacacttct tcaagaaca attcaagacc gtgtcaacc ctcaacggcc
 841 ggagagtga gatlccac gaccacgagc gtgtcgataa aaaagaacc gtaacaaaa
 901 agaaaaaacc caat taaat aglttacat tgtctattgg ttgtttaga accctaatta
 961 gcttgttaa actaataatc tctatgtag tactgtgtg ttgtttacga ctgatatac
 1021 gatttccaaa aaaaaaaaa aaaaaa

We have concluded that CAH1 has an N-terminal signal peptide that targets the protein to the ER. The results of our studies of the mechanism further suggests that the mature stroma (chloroplast/plastid stroma) CAH1 protein is N-glycosylated and also that this protein is not the only glycosylated protein in Arabidopsis chloroplasts/plastids. Interestingly, the occurrence of potential N-glycosylation sites is not the only common feature among the glycosylated stroma proteins. Based on comparisons with other such proteins we conclude that the C-terminal, which is highly hydrophilic and charged, including lysine residues, seems to be important for the import mechanism of these proteins into the plastid.

A typical expression construct based on the sequences from the CAH1 system could contain the following parts:

- A selectable marker to facilitate selection of the transgenes under an appropriate promoter.
- A promoter to drive the expression of the gene of interest. This promoter could be chosen among known promoters or promoters optimised for use in plant systems, e.g. a constitutive promoter such as the CmV35S promoter (or variants of it) or it could be an inducible promoter such as an heat inducible promoter. For use of certain inducible

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promoters an introduced transcription factor (natural or constructed) is required and such transcription factor could be under the control of different promoters. The promoter could also be tissue specific, such as seed specific, leaf specific, etc, and or specifically expressed at different times, developmental, seasonally, diurnal.

- A 5' un-translated region can be added to the construct in order control the translational initiation efficiency and transcript stability.
- The protein coding part of the construct starts with an ER signal peptide, e.g an approximately 24 amino acids (aa) sequence from the CAH1 gene described below followed by an chloroplast transit peptide like sequence (for example aa 25-75 of the CAH1 gene). After this an endoprotease, or other site can be added to facilitate the removal of any remaining signal sequences after processing. Then the sequence coding for the desired protein to be expressed is inserted. Additions and removals of glycosylation sites can be performed in this sequence depending on the need for glycosylation of the final product. Then the same or a different endoprotease or similar site can be added before the c-terminal sequence consisting of the last 61aa of the CAH1 gene.
- A 3' UTR and a terminator can be added which would facilitate transcript termination, poly adenylation and transcript stability.
- After harvest the protein or the chemical compound produced have to be purified this is facilitated by the fact that one first can purify the chloroplasts and then the protein from the chloroplasts have to be purified. The protein could also be purified from for example a whole leaf.

In a reduction to practice experiment transient expression of *Arabidopsis* CAH1 fused to the green fluorescent protein (GFP) was performed in *Arabidopsis* and tobacco cells. As expected, the expressed GFP protein (negative control) was distributed uniformly in the cytosol and in the nucleus whereas the plastid control (transit sequence of Rbcs fused to GFP) was targeted to the chloroplast. Transient expressions of the complete CAH1 protein were then performed. Plastid localization was obtained with the CAH1 protein when GFP was fused to its C-terminus. For further examination of the domain required for plastid localization of the CAH1 protein, we generated several versions of the CAH1 protein and transient expressions of corresponding GFP fusions were tested into *Arabidopsis* and BY2 tobacco cells. The signal peptide of CAH1 directed GFP to the ER in *Arabidopsis* protoplasts.

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Example of the construct where the GFP is the reporter gene that should be exchanged with any protein of interest), this construct will target the GFP to the plastids.



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1      mkimmmiklc ffsmslicia padaqtegvv fgykgkngpn qwghlnphft tcavgklqsp
61     idiqrriqify nhklnsihre yyftnatlvn hvcnvamffg egagdviien knyt
      + GFP (or the gene to be produced)+
224    ilgkvrms mskeqvellr
241    spldtsfknn srpcqplngr rvemfhdhcr vdkketgnkk kkpn
  
```

Accordingly a recombinant construct of the invention comprises an ER signal sequence and a plastid signalling sequence. Examples of such sequences are found in CAH1 (sequence given above) and analogues.

The predicted minimal ER signalling sequence has been found to be an N-terminal amino acid sequence of about 24 amino acids. Examples of ER signalling sequences include, without limitation:

MKIMMMIKLCFFSMSLICIA PADA	CAH1 <i>Arabidopsis</i>
MAASHGNAIFVLIICTFLPSLAC	CAH1 Rice
MAARIGIFS FVAVLLSISAFSSA	Ribophorin I <i>Arabidopsis</i>

In addition to the ER signal sequence a sequence necessary for localisation to the stroma in the chloroplast, i.e. to the next and final destination in the plant, a C-terminal sequence of the CAH1 protein or a functional analogue is required. At present we believe that such a minimal sequence from the CAH1 protein system comprises a 12-15 amino acid sequence or a functional analogue. This sequence could probably also be located directly after the ER signal, even if it according to the at present preferred embodiment is located downstream the desired protein. Examples of such sequences include, without limitation the following sequences or functional derivatives thereof. Such functional derivatives are characterised by comprising 3-4 lysines in a row.

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KKETGNKKKKPN

RFWGKKKRRSSP

TGKKKKKTYLP

CAH1 *Arabidopsis*

CAH1 Rice

Ribophorin 1 *Arabidopsis*

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